Engineering increased triacylglycerol accumulation in *Saccharomyces cerevisiae* using a modified type 1 plant diacylglycerol acyltransferase

Michael S. Greer · Martin Truksa · Wei Deng · Shiu-Cheung Lung · Guanqun Chen · Randall J. Weselake

Abstract Diacylglycerol acyltransferase (DGAT) catalyzes the acyl-CoA-dependent acylation of *sn*-1,2-diacylglycerol to produce triacylglycerol (TAG). This enzyme, which is critical to numerous facets of oilseed development, has been highlighted as a genetic engineering target to increase storage lipid production in microorganisms designed for biofuel applications. Here, four transcriptionally active *DGAT1* genes were identified and characterized from the oil crop *Brassica napus*. Overexpression of each *BnaDGAT1* in *Saccharomyces cerevisiae* increased TAG biosynthesis. Further studies showed that adding an N-terminal tag could mask the deleterious influence of the DGATs' native N-terminal sequences, resulting in increased in vivo accumulation of the polypeptides and an increase of up to about 150-fold in in vitro enzyme activity. The levels of TAG and total lipid fatty acids in *S. cerevisiae* producing the N-terminally tagged BnaDGAT1.b at 72 h were 53 and 28 % higher than those in cultures producing untagged BnaA.DGAT1.b, respectively. These modified DGATs catalyzed the synthesis of up to 453 mg fatty acid/L by this time point. The results will be of benefit in the biochemical analysis of recombinant DGAT1 produced through heterologous expression in yeast and offer a new approach to increase storage lipid content in yeast for industrial applications.

Keywords Yeast · DGAT · Storage lipid synthesis · *Brassica napus* · Biofuel

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Introduction

Triacylglycerol (TAG) serves as feedstock for the production of biodiesel, which is composed of fatty acid alkyl esters (Knothe 2005). Biodiesel derived from plant TAG is nearly neutral with regard to production of carbon dioxide because the hydrocarbon chains of TAG (and biodiesel) are derived from the photosynthetic-driven capture of carbon dioxide (Durrett et al. 2008; Lackey and Paulson 2011). There have been, however, serious concerns expressed about plant-derived biodiesel leading to increased food prices or environmental damage due to increased oilseed production (Durrett et al. 2008). Currently, seeds of oleaginous crops serve as the major feedstock for biodiesel production, but reduced land requirements, weather independence, and lower labor inputs have made microorganisms such as yeast an attractive alternative (Li et al. 2008). Although yeasts do not rely on photosynthesis for carbon, these microorganisms can be engineered to utilize various carbon sources such as those found in agricultural waste (Yu et al. 2013).

Metabolic engineering of yeast to feed acyl chains into TAG can create a "pull" effect on fatty acid synthesis, leading to greater cellular fatty acid content (Tai and Stephanopoulos 2013). As a result, diacylglycerol acyltransferase (DGAT) has been identified as a promising target for engineering increased

oil content (Liang and Jiang 2013; Runguphan and Keasling 2014). DGAT is a membrane-bound enzyme which catalyzes the acyl-CoA-dependent acylation of *sn*-1,2-diacylglycerol (DAG) to produce TAG (Liu et al. 2012). Plants, animals, and some yeast species possess two unrelated forms of DGAT referred to as DGAT1 and DGAT2 (Liu et al. 2012; Lung and Weselake 2006). DGAT1 enzymes belong to a family of membrane-bound O-acyltransferases and have a typical mass of about 60 kDa. DGAT1s have been predicted to have between 6 and 12 transmembrane domains (McFie et al. 2010). In contrast, DGAT2 belongs to a family of DGAT2/acyl-CoA:monoacylglycerol acyltransferase (McFie et al. 2010), which are typically smaller than DGAT1 enzymes. DGAT2 has been demonstrated to have four transmembrane domains (Liu et al. 2011). It has been observed in plants that DGAT1 and DGAT2 localize to different subdomains of the endoplasmic reticulum, suggesting these enzymes may have evolved non-redundant roles (Shockey et al. 2006). Coding DNA sequences (CDS) of eukaryotic DGAT of either family have been overexpressed both in plants and microorganisms as a means of increasing TAG content (Andrianov et al. 2010; Kamisaka et al. 2007; Tai and Stephanopoulos 2013; Taylor et al. 2009; Yu et al. 2013).

Although there are numerous species of oleaginous yeasts which naturally produce high TAG content, *Saccharomyces cerevisiae* features numerous advantages for engineering increased oil production, including established genetic resources and molecular tools, availability of a large assortment of deletion strains, short generation time, ease of culturing, and a proven track record in industry (Runguphan and Keasling 2014; Tang et al. 2013). Additionally, plant lipid research can directly contribute to the metabolic engineering of *S. cerevisiae* as this species is commonly used as model system for the study of liposynthetic enzymes from plants. The first demonstration of a plant DGAT used to increase oil content in *S. cerevisiae* was that of Bouvier-Navé et al. (2000) where the yeast was transformed with a CDS encoding an *Arabidopsis thaliana* DGAT. The primary focus of that study, however, was to functionally evaluate the plant DGAT. Contrary to *S. cerevisiae* which possesses only DGAT2, DGAT1 has been shown to play a predominant role in TAG synthesis in plants (Liu et al. 2012; Li et al. 2010).

Here, we report on the identification of four actively transcribed *Brassica napus* DGAT1 (*BnaDGAT1*) genes and use of their CDS in increasing oil content in *S. cerevisiae*. We also identified that a polymorphism in the second codon of the *BnaDGAT1* CDS which can substantively affect the accumulation of the recombinant BnaDGAT1 polypeptides resulting in large differences in TAG production. We further demonstrate that modification of the N-terminal sequence of BnaDGAT1 as a simple method of circumventing the effects of native sequences of BnaDGAT1 when expressed in yeast, which does not impact cellular growth rates. This modification

represents an additional tool to those currently available to produce greater TAG content in *S. cerevisiae*.

Materials and methods

Yeast culture conditions

Two yeast strains, a TAG-deficient quadruple mutant *S. cerevisiae* H1246 (*MATα are1-Δ::HIS3 are2-Δ::LEU2 dga1-Δ::KanMX4 lro1-Δ::TRP1 ADE*) and its parental strain *S. cerevisiae* SCY 62 (*MAT a ADE2 can 1-100 his3-11,15 leu2-3 trp1-1 ura3-1*), were used in this study (Sandager et al. 2002). Cultures of *S. cerevisiae* H1246 were initiated in 2 % (*w/v*) glucose synthetic liquid media lacking uracil (0.67 % (*w/v*) yeast nitrogen base and 0.2 % (*w/v*) synthetic complete dropout mix (SC synthetic minimal media). After overnight growth, cultures were inoculated into synthetic media containing 2 % (*w/v*) galactose and 1 % (*w/v*) raffinose to an optical density of 0.4. Cultures were rotated at 250 rotations/min at 30 °C. *S. cerevisiae* SCY 62 was grown similarly, unless otherwise indicated, with the exception that they were all initiated in synthetic media containing 1 % (*w/v*) raffinose as the carbon source.

Cloning of BnaDGAT1 cDNA and genes

A *cDNA* library representing a full range of seed developmental stages was constructed using the double haploid *B. napus* line DH12075 (Séguin-Swartz et al. 2003). With information obtained from the *B. napus* EST database (National Research Council of Canada, Saskatoon, Saskatchewan), the library was used to isolate full length *BnaDGAT1* cDNA using the Marathon cDNA amplification kit (Clontech) and primers indicated in Table 1-A. Genomic DNA was isolated from *B. napus* line DH12075 seedlings and used for isoform-specific *BnaDGAT1* amplification (Table 1-B). Amplified products were then cloned into the pCR4-TOPO TA vector (Invitrogen) as per the manufacturer's instructions. The 3-kb genes were sequenced (Table 1-C or manufacturer's primers), and results were assembled using SeqMan (DNAstar).

Generation of yeast expressing BnaDGAT1

Full length *BnaDGAT1* CDS were amplified by PCR (Table 1-C) and cloned into the pYES2.1/V5-His TOPO yeast expression vector (Invitrogen), under the control of the *Gal1* promoter and with the addition of a 3′ V5 epitope. *BnaDGAT1* fragments were amplified by PCR and used as templates to construct full length chimera constructs by the overlapping PCR method (Table 1-D). Chimera constructs were cloned into the pYES2.1/V5-His

Table 1 Primers used for amplification of DGAT1 identification, sequencing, and construct design

Construct	Oligonucleotide sequences (5′-3′ orientation)
A. Primers used for BnaDGAT1 cDNA cloning	
BnaC.DGAT1.a	TCTGGAGGCGTCACTATGC, CCCGCTTCTCCACCG
BnaA.DGAT1.a	TCTGGAGGCGTCACTATGC, CTTCTCCGCCGCCTC
BnaA.DGAT1.b	TCGCTGTACCGCCGAC, TAAAACCTTACATCGCCTCCG
BnaC.DGAT1.b	AGGCGTCGCTGTACCGA, TACATCGCCGTTTCCTCCT
B. Primers used for genomic BnaDGAT1 sequence isolation	
BnaC.DGAT1.a	TTGGATTCTGGAGGCGTCACTATG, ACATCTATGACATCTTTCCTTTGC
BnaA.DGAT1.a	TTGGATTCTGGAGGCGTCACTATG, ACATCTATGACATCTTTCCTTTGC
BnaA.DGAT1.b	TTGGATTCTGGAGGCGTCGCTGTA, TGTGGAGTAAAAAGTCCTTCTCAG
BnaC.DGAT1.b	TTGGATTCTGGAGGCGTCGCTGTA, TGTGGAGTAAAAAGTCCTTCTCAG
C. Primers used for subcloning BnaDGAT1s	
BnaC.DGAT1.a	ACCATGGAGATTTTGGATTCTGGAGGCG,TGACATCTTTCCTTTGCGGTTC
BnaA.DGAT1.a	ACCATGGAGACTTTGGATTCTGGAGGCG, TGACATCTTTCCTTTGCGGTTC
BnaA.DGAT1.b	ACCATGGCGGTTTTGGATTCTGGAGGCG, GGACATGGATCCTTTGCGGTTC
BnaC.DGAT1.b	ACCATGGCGGTTTTGGATTCTGGAGGCG, GGACATGGATCCTTTGCGGTTC
D. Primers used for chimeric DGAT1s	
BnaC.DGAT1.a(NT)::BnaA.DGAT1.b(NTD)	ACCATGGAGATTTTGGATTCTGGAGGCG,GATACGTAAACCTTACATCGCCT CCCGCTTCTCCACCGGGATTCCCTAATT, TCTCCACCGGGATTCCCTAATT, AATTAGGGAATCCGGTGGAGAAGCGGGAGGCGATGTAAGGTTTACGTATC
BnaA.DGAT1.b(NT)::BnaC.DGAT1.a(NTD)	ACCATGGCGGTTTTGGATTCTGGAGGCG, TGTACCTTACATCCACGTTTCCC CCGGCGGATTCCCTAGTTTCGGCATCT, AGATGCCGAAACTAGGGAATCC GCCGGGGGAAACGTGGATGTAAGGTACA, TGACATCTTTCCTTTGCGGTTC
E. Primers used for site-directed mutations	
BnaC.DGAT1.a(E2A)	ACCATGGAGATTTTGGATTCTGGAGGCGTCAC, TGACATCTTTCCTTTGCGGTTC
BnaA.DGAT1.b(A2E)	ACCATGGAGGTTTTGGATTCTGGAGGCGTCGC, GGACATGGATCCTTTGCGGTTCAT
BnaA.DGAT1.b(AV/EI)	ACCATGGAGATTTTGGATTCTGGAGGCGTCGC, GGACATGGATCCTTTGCGGTTCAT
F. Primers used for N-terminal tagging	
NT::BnaC.DGAT1.a	TAAAATGCGGCCGCATGGAGATTTTGGATTCTGGAG,TAAAATTCTAGATGACAT CTTTCCTTTGCGGTTC
NT::BnaA.DGAT1.b	TAAAACGCGGCCGCATGGCTGTTTTGGATTCTGG,TAAATTCTAGAGGACATGGAT CCTTTGCGGTTC

TOPO yeast expression vector (Invitrogen) as described above. *BnaDGAT1* coding DNA sequences were also amplified by PCR with forward primers designed to introduce site-directed mutations at the +5 and +7 nucleotides (Table 1-E) before cloning into pYES2.1/V5-His TOPO (Invitrogen). Restriction sites *Not*I and *Xba*I were added to *BnaDGAT1* CDS by PCR amplification (Table 1-F). Resulting PCR products were digested and cloned in frame into pYES2/NT-B (Invitrogen) to produce NT-DGAT1. The N-terminal tag of this vector is composed of 6× polyhistidine linked to an "Xpress" epitope. It and all other constructs were cloned in frame with 3′ V5 epitope coding sequences and were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) to ensure the fidelity of the coding sequences. Vectors were transformed into the *S. cerevisiae* strains by polyethylene glycol-mediated transformation (Gietz and Schiestl 2007).

Analysis of neutral lipid content in yeast

Nile red analysis of TAG accumulation was performed as described previously (Siloto et al. 2009a) using either a Flourskan Ascent (Thermo Electron Company) or a Synergy H4 hybrid reader (Biotek). In brief, 95 μl of yeast culture was incubated with 5 μl of 0.8 μg/mL Nile red solution (suspended in methanol). Fluorescent emission was assayed before and 5 min after addition of Nile red solution. Change in fluoresce over optical density ($\Delta F/OD_{600}$) was used as a measure of cellular neutral lipid content. Three technical replicates of three biological replicates were performed for each BnaDGAT1 isoform and construct.

Preparation of microsomes and Western blotting

Microsomal fractions were isolated from *S. cerevisiae* H1246 cultures expressing *DGAT1* constructs according to Siloto

et al. (2009b). For Western blotting, 25 μg of isolated protein was separated by 10 % denaturing SDS-PAGE. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes which were then blocked with 2 % milk fat before incubation with V5-HRP-conjugated antibody (1:10,000). BnaDGAT1 content was visualized using ECL Advance Western Blotting Detection Kit (Amersham) with the aid of a variable mode imager (Typhoon Trio+, GE Healthcare).

In vitro DGAT activity assays

DGAT assays were conducted in a similar fashion to the procedure described by Byers et al. (1999). Microsomes containing recombinant BnaDGAT1 were incubated in the presence of 200 mM Hepes-NaOH (pH 7.4), 3.2 mM $MgCl_2$, 333 μM sn-1,2-dioleoyl-glycerol dispersed in 0.2 % Tween-20 (Avanti Polar Lipids), and 15 μM [1-^{14}C]oleoyl-CoA (55 μCi/μmol) (PerkinElmer). DGAT reactions were allowed to proceed at 30 °C for 2–5 min before quenching with sodium dodecyl sulfate. Entire reactions were then spotted onto G25 silica plates, and TAGs were isolated using 80:20 hexane/ether (v/v) as a mobile phase. After air-drying, silica containing TAG spots were identified using an imagine plate (Fujifilm) in combination with a Typhoon tri+ variable mode imager (Amersham Biosciences). Identified spots were scraped from the thin-layer chromatography (TLC) plate, combined with Ecolite scintillation cocktail (MP Biomedicals) and radioactivity quantified using an LS 6500 multi-purpose scintillation counter (Beckman Coulter).

Yeast fatty acid analysis

Wild-type S. cerevisiae cells were harvested 72 h post-induction, flash frozen, and freeze dried. Approximately 60 mg of sample was rehydrated, and lipids were extracted using the Folch method, with minor modifications (Christie and Han 2010). Isolated lipids were resuspended in chloroform and split into two equal portions: one portion was to TLC isolation of TAG (80:20:1 hexane/diethyl ether/acetic acid mobile phase), and the remaining portion was used to determine total lipid content. TAG spots on TLC plates (silica G25) were identified using primulin staining. Both isolated TAG and dried down total lipid samples were derivatized by incubation in 2 mL methanolic HCl for 3 h at 80 °C. Reactions were quenched with addition of 2 mL of saline, and methyl esters were isolated with two hexane extractions. Isolated fatty acid methyl esters were separated along a 30-m DB23 column (Agilent Technologies) using a 6896 N network GC system (Agilent Technologies) and quantified using a 5975 inert XL mass selection detector (Agilent Technologies). Triheptadecanoin and heneicosanoic methyl ester (Nu-Chek Prep.) were used as external and internal controls, respectively. Three biological replicates were tested for each construct.

Visualization of yeast fat pads

S. cerevisiae H1246 cultures were grown as described above for 24 h. Cells were lysed and centrifuged as described for the isolation of microsomes. The resulting fat pads in the centrifuge tubes were photographed using a Canon EOS 60D camera (with a Canon EF 100 mm f/2.8 Macro USM lens).

Statistical analysis

Analysis in vivo TAG accumulation and fatty acid composition of yeast-producing DGAT1 enzymes was performed using one-way analysis of variance (ANOVA; SAS 9.3, SAS Institute Inc), with $\alpha=0.05$.

Nucleotide accession numbers

Brassica DGAT1 gene sequences were awarded the following accession numbers: BnaA.DGAT1.a (JN224474), BnaC.DGAT1.a (JN224473), BnaA.DGAT1.b (JN224475), and BnaC.DGAT1.b (JN224476).

Results

Identification of BnaDGAT1 genes and coding sequences

Four different DGAT1 mRNA sequences were identified in cDNA amplified from developing B. napus line DH12075 seeds (Séguin-Swartz et al. 2003), and the presence of the four BnaDGAT1 genes in the B. napus genome was confirmed by full gene sequencing and Southern blot analysis (Supplemental Fig. S1). The genes were named according to the nomenclature suggested for Brassica species (Ostergaard and King 2008). B. napus is an allotetraploid containing two (A and C) of the three ancestral Brassica genomes (Liu and Wang 2006). Two of the BnaDGAT1s, BnaA.DGAT1.a (GenBank ID: JN224474) and BnaA.DGAT1.b (GenBank ID: JN224475), displayed high sequence homology to DGAT1 genes identified in B. rapa, a species containing only the Brassica A genome (BRAD 2011). The coding sequence of BnaA.DGAT1.b was previously reported by our group (GenBank ID: AF164434.1) (Nykiforuk et al. 1999), whereas BnaA.DGAT1.a has not been previously reported. The remaining two BnaDGAT1 genes showed high sequence homology to DGAT1 nucleotide sequences present in EST libraries derived from B. oleracea, which possesses only the Brassica C genome. The mRNA sequence of BnaC.DGAT1.a (Genbank ID: JN224473) was registered into the NCBI database by Brown et al. (1998) as GenBank ID: AF251794.1, whereas BnaC.DGAT1.b (Genbank ID: JN224476) has not been previously reported. All four genes appear to have

similar organization, common to *DGAT1* genes, with a large first exon followed by 15 smaller exons.

The *BnaDGAT1* coding sequences could be divided into two groups based on homology (Fig. 1a), with *BnaC.DGAT1.a* and *BnaA.DGAT1.a* falling into one clade and *BnaA.DGAT1.b* and *BnaC.DGAT1.b* into a second clade. The first and second clades share 98.6 and 96.2 % pairwise identity, respectively, compared to 91.0 % pairwise identity for all four BnaDGAT1 amino acid sequences. The amino acid sequence differences among the encoded enzymes reside primarily in the hydrophilic polypeptide segment encoded by the first exon (74.2 % amino acid pairwise identity) (Fig. 1b).

Functional characterization of BnaDGAT1 coding sequences

To initially characterize the four BnaDGAT1, their coding sequences were first expressed in the quadruple knockout *S. cerevisiae* strain H1246 ($\Delta are1$ $\Delta are2$ $\Delta lro1$ $\Delta dga1$) which lacks the genes necessary to synthesize neutral lipids (Sandager et al. 2002). As a result, the neutral lipid content of these cultures directly relates to the amount of TAG produced by the introduced *DGAT1* CDSs. Nile red fluoresces at a unique wavelength in the presence of neutral lipids, and thus, under the above conditions, provides for a rapid method to quantify TAG produced by the catalytic action of recombinant DGATs (Siloto et al. 2009a). Using this method, it was demonstrated that all four *BnaDGAT1* CDS encoded active DGAT enzymes. Large differences in TAG synthesis rates were observed, however, despite the high degree of homology shared between these coding sequences. Yeast-producing BnaA.DGAT1.a generated far greater TAG content compared to yeast-producing BnaA.DGAT1.b (Fig. 2a). Similarly, cultures producing BnaC.DGAT1.a generated significantly more TAG than those producing BnaC.DGAT1.b. Western blot of microsomal protein extracts revealed that the divergent in vivo DGAT activities were largely a product of differential accumulation of the polypeptides (Fig. 2b). Differences in DGAT1
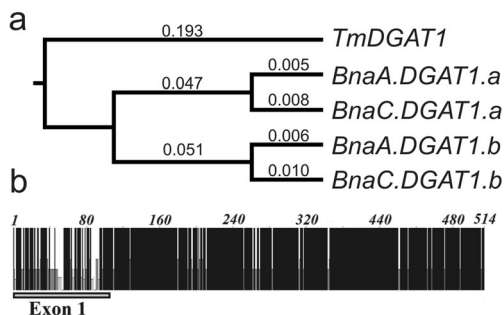

Fig. 1 BnaDGAT1 sequence homology. **a** Phylogenetic tree of *BnaDGAT1* coding sequences. *Numbers* indicate substitutions per site. **b** Homology alignment of BnaDGAT1 polypeptides. *Higher bar height and darker shade* indicate higher amino acid residue identity consensus. *Shaded rectangle* indicates amino acid residues encoded by the first exon of *BnaDGAT1*
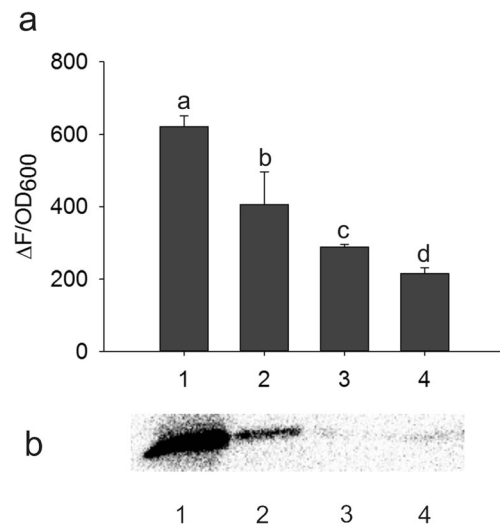

Fig. 2 In vivo TAG (**a**) and BnaDGAT1 (**b**) accumulation in *S. cerevisiae* strain H1246 cultures producing different forms of BnaDGAT1. Nile red readings, and microsomal protein extractions were performed with cultures of similar optical densities (OD_{600}) 24 h post-induction. *1* BnaC.DGAT1.a, *2* BnaA.DGAT1.a, *3* BnaA.DGAT1.b, *4* BnaC.DGAT1.b. *Error bars* indicate ±1 standard deviation. *Letters* indicate statistical grouping

content appeared to be of a larger scale than those observed for the TAG content of these cultures. This discrepancy may result from the yeast being incapable of providing sufficient substrates for use by the highly accumulating DGAT1s, leading to proportionately smaller returns in TAG contents over time.

Following these initial results, we subsequently observed that expressing coding sequences of two candidate BnaDGAT1 variants (*BnaC.DGAT1.a* and *BnaA.DGAT1.b*) in frame with 5′ epitopes from the pYES2.1-NT vector produced enzymes (NT::BnaDGAT1) which accumulated significantly more neutral lipids than their equivalents with no N-terminal tag (Fig. 3a). As well, in vitro assays with radiolabeled acyl-CoA and exogenous DAG indicated that the tagged polypeptides displayed high enzyme-specific activity (Fig. 3b). A 2-min assay could use as little as 150 ng of microsomal protein to produce a relatively large quantity of TAG. The high DGAT activity observed was almost completely dependent on exogenous DAG; enzyme activity was reduced by approximately 98 % when exogenous DAG was not made available. Under optimized conditions, the specific activities of microsomal DGAT for oleoyl-CoA in the presence of *sn*-1,2-diolein were 17.4 and 6.76 nmol TAG min^{-1} mg protein^{-1} for NT::BnaC.DGAT1.a- and NT::BnaA.DGAT1.b-producing cultures, respectively. These activities represented increases of approximately 16- and 149-fold, respectively, over their untagged equivalents. The impact of expressing BnaDGAT1 enzymes with and without the N-terminal tag is significant enough that differences in fat pad accumulation can be observed visually (Fig 3c).
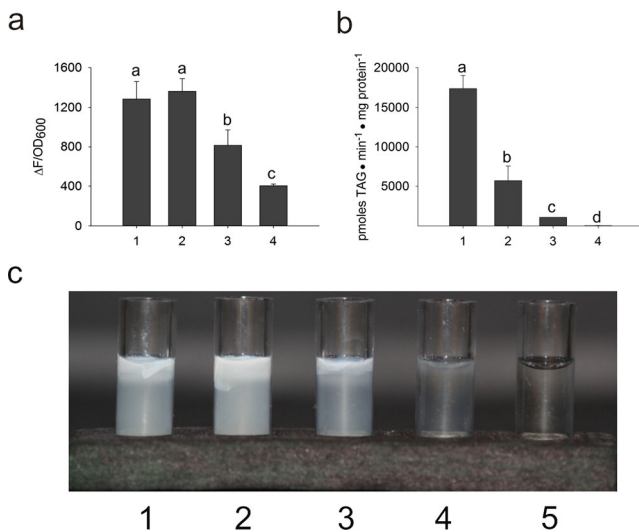
Fig. 3 Effect of N-terminal modification of BnaDGAT1 on lipid accumulation and microsomal DGAT activity for recombinant enzymes produced in *S. cerevisiae* H1246. Nile red analysis of in vivo lipid accumulation (**a**) was performed on cultures of similar optical densities (OD_{600}) 24 h post-induction. In vitro DGAT enzyme assays (**b**) were conducted using [1-^{14}C] oleoyl-CoA in the presence of exogenous *sn*-1,2-diolein. *Error bars* indicate ±1 standard deviation. Fat pads (**c**) were isolated from 50 mL cultures 24 h post-induction from cultures grown at 30 °C, 250 rpm. *1* N-terminally tagged BnaC.DGAT1.a (NT::BnaC.DGAT1.a); *2* NT::BnaA.DGAT1.b; *3* BnaC.DGAT1.a; *4* BnaA.DGAT1.b. *5* LacZ. *Letters* indicate statistical grouping

To understand how the N-terminal tag was increasing DGAT1 activity, we attempted to elucidate why the enzymes without N-terminal tags displayed such different in vivo activities. We hypothesized that the differences in BnaDGAT1 activity may be a product of the divergent amino acid sequences present in their N-terminal domain, as the remainder of their polypeptide sequences displayed very high similarity. To test this hypothesis, yeast cultures expressing chimeric versions of the candidate CDS with interchanged N-terminal domains (i.e., BnaC.DGAT1.a$_{(N-Terminus)}$::BnaA.DGAT1.b$_{(N-Terminus\ Deleted)}$ and BnaA.DGAT1.b$_{(NT)}$::BnaC.DGAT1.a$_{(NTD)}$) were subjected to Nile red analysis. The N-terminal domains of these chimeras represent the amino acids encoded by the first exons of their parental gene (Fig. 1b). TAG content in cultures producing these chimeric proteins accumulated to levels equal to the native *B. napus* enzyme with which they shared N-terminal homology (Fig. 4a). BnaC.DGAT1.a$_{(NT)}$::BnaA.DGAT1.b$_{(NTD)}$ generated an equal amount of TAG to cultures producing BnaC.DGAT1.a, which was up to twofold more TAG than that generated by cultures producing BnaA.DGAT1.b. The reciprocal was also observed for cultures producing BnaA.DGAT1.b$_{(NT)}$::BnaC.DGAT1.a$_{(NTD)}$, which generated far less TAG than BnaC.DGAT1.a cultures. Western blots performed on microsomal protein extracts from the yeast cultures demonstrated the TAG content phenotype was dependent upon the variant form of the N-terminal region of these DGATs (Fig. 4b).

In silico analysis of the N-terminal domains of the BnaDGAT1 CDS identified a potential phosphorylation site (BnaA.DGAT1.b$_{S30}$) and a potential ubiquitination site (BnaA.DGAT1.b$_{R27}$) which theoretically could lead to the differential accumulation of the enzymes. Replacement of these amino acids through site-directed mutagenesis, however, had no effect on the enzyme's activity, suggesting the accumulation of the enzyme was not affected (data not shown). Mutation of the second amino acid residue in the BnaDGAT1 polypeptide sequences, conversely, was shown to have a large effect on the enzymes' activity (Fig. 4c). Yeast-producing BnaC.DGAT1.a with its second amino acid residue (glutamate) converted to an alanine residue produced significantly less oil than cultures producing BnaC.DGAT1.a. The reverse was observed for cultures producing BnaA.DGAT1.b with its second amino acid residue (alanine) replaced with a glutamate residue, which produced significantly more oil than cultures producing unmodified BnaA.DGAT1.b. Replacing both the second and third amino acid residues of BnaA.DGAT1.b led to even greater oil accumulation. Mutation of these amino acid residues was also shown to dramatically alter the accumulation of these polypeptides in vivo (Fig. 4d) as well. Interestingly, BnaC.DGAT1.a$_{(E2A)}$ catalyzed the production of less oil than BnaA.DGAT1.b, despite it apparently accumulating to a higher degree. This may suggest that mutating the second amino acid can affect both DGAT accumulation and its enzymatic activity. It is possible, however, that some discrepancy can be expected between Western blot and Nile red analysis as these assays are performed at different time points in the yeast's growth
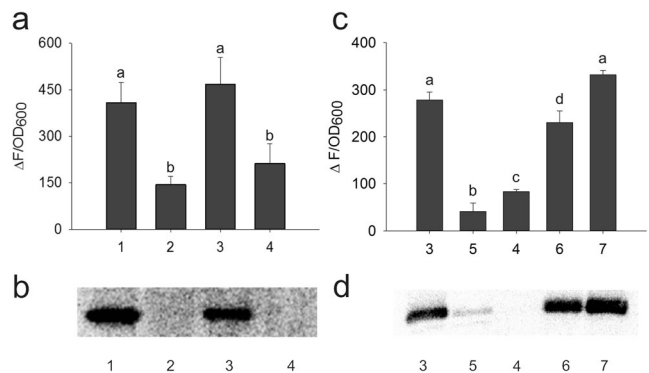

Fig. 4 Impact of BnaDGAT1 N-terminal form on enzyme activity and accumulation in *S. cerevisiae* strain H1246. In vivo neutral lipid (**a**) and BnaDGAT1 accumulation (**b**) in H1246 *S. cerevisiae* cultures expressing chimeric DGAT1 coding sequences. In vivo neutral lipid (**c**) and BnaDGAT1 accumulation (**d**) in H1246 *S. cerevisiae* cultures expressing mutated *BnaDGAT1* coding sequences. Nile red readings and microsomal protein isolations were performed on cultures at similar optical densities (OD_{600}) 24 h post-induction. *Error bars* indicate ±1 standard deviation. *1* BnaC.DGAT1.a$_{(N-Terminus)}$::BnaA.DGAT1.b$_{(N-Terminus\ Deleted)}$, *2* BnaA.DGAT1.b$_{(NT)}$::BnaC.DGAT1.a$_{(NTD)}$, *3* BnaC.DGAT1.a, *4* BnaA.DGAT1.b, *5* BnaC.DGAT1.a $_{(\Delta E2A)}$, *6* BnaA.DGAT1.b$_{(\Delta A2E)}$, *7* BnaA.DGAT1.b$_{(\Delta AV/EI)}$. *Letters* indicate statistical grouping

curve. In either scenario, however, producing BnaDGAT1 enzymes with an N-terminal epitope in yeast appears to mask the impact of deleterious nucleotide or amino acid residues present at leading terminus of these enzymes, leading to increased enzyme production and, subsequently, increased in vivo TAG synthesis.

Potential use of tagged BnaDGAT1 for industrial biofuel applications

Placement of an N-terminal tag onto DGAT1 enzymes represents a simple method of increasing in vivo DGAT activity which could easily be combined, with many other methods shown to increase yeast cellular TAG content. To provide insight as to how useful these higher accumulating DGAT1 enzymes could be for yeast based fatty acid production and biofuel applications, the performance of the enzymes was followed when produced in wild-type S. cerevisiae cells (SCY 62). First, the impact of increasing BnaDGAT1 content upon cellular growth was monitored. As shown in Fig. 5a, cultures expressing BnaDGAT1 constructs, with or without coding segments for N-terminal epitopes, grew identically to cultures expressing LacZ. Having ensured N-terminal tagging did not impede yeast growth, the accumulation of neutral lipids was then followed over time in cultures expressing NT::BnaA.DGAT1.b (Fig. 5b). Cultures producing this enzyme were observed to immediately produce significantly more neutral lipid relative to both LacZ and BnaA.DGAT1.b-expressing cultures. At approximately 28 h, the yeast cells reached their maximal content of neutral lipids. At this point, the NT::BnaA.DGAT1.b cultures had 115 % greater neutral lipid content than cultures expressing LacZ and 54 % more neutral lipid than cultures expressing BnaA.DGAT1.b. The increased cellular neutral lipid levels of NT::BnaA.DGAT1.b-

expressing cultures were not limited to a particular window of time and consistently remained higher than the BnaA.DGAT1.b and LacZ-expressing cultures.

To complement the Nile red analysis of wild-type cultures expressing NT:BnaA.DGAT1.b, GC-MS was used to analyze the fatty acid content of TAG and total lipid from these cultures 72 h post-induction. These results were quite consistent with data obtained from Nile red analysis for this time point. Expression of BnaA.DGAT1.b increased the cellular TAG content of yeast by 50 %, and total lipid by 30 %, relative to cultures expressing LacZ (Fig. 6a). Modification of the N-terminus of BnaA.DGAT1.b, however, resulted in significantly greater lipid content. Wild-type cultures expressing NT::BnaA.DGAT1.b produced 9.9 % total lipid and 6.4 % TAG, respectively, on a dry cell weight basis. These results translate into a 130 % increase in TAG content, and a 67 % increase in total lipid, relative to cultures expressing only LacZ. These levels of TAG and total lipid fatty acids were 53 and 28 % higher than those observed in cultures producing untagged BnaA.DGAT1.b. The mass of fatty acids not esterified into TAG molecules (i.e., sterol esters, polar lipids) appeared to be equivalent in all of the samples, suggesting the increase in total lipids was solely a product of increased TAG production.

Interestingly, the fatty acid composition of lipid fractions from yeast cells appeared to be influenced by the degree to which BnaA.DGAT1.b accumulated. Cultures producing NT::BnaA.DGAT1.b generated TAG that contained 11.2 % less unsaturated fatty acids on an absolute basis than cultures expressing LacZ (Fig. 6b). Relative to the LacZ control, however, cultures producing BnaA.DGAT1.b generated TAG reduced in unsaturated fatty acids by only 6.4 %. The changes in TAG fatty acid composition were mirrored in the total fatty acid composition (Fig. 6c). Increasing
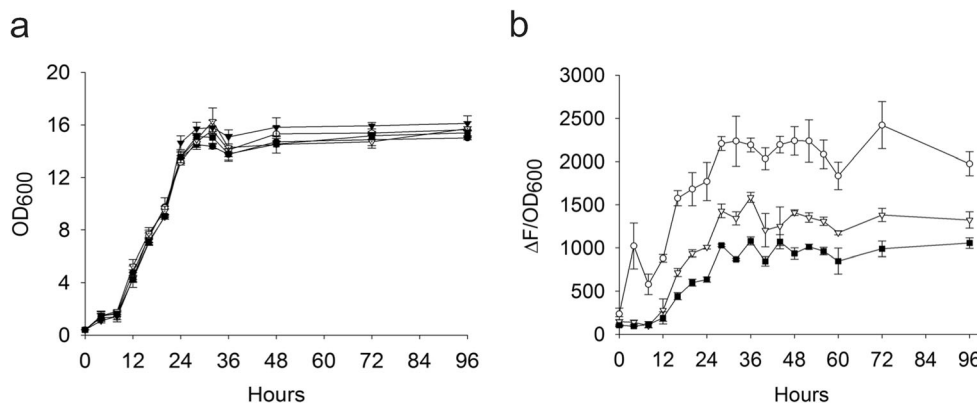

Fig. 5 Growth and relative neutral lipid content of wild-type S. cerevisiae producing N-terminally modified BnaDGAT1 enzymes. a Growth rates of cultures producing BnaDGAT1 polypeptides. Determination of optical densities began with inoculation of 50 mL of induction media to OD_{600} of 0.4. Cultures were grown at 30 °C in 250-mL Erlenmeyer flasks, rotated at 250 rotations/min. Accumulation of neutral lipids over time (b) in

cultures expressing BnaDGAT1 constructs. Error bars indicate ±1 standard deviation. Black circle: N-terminally tagged BnaC.DGAT1.a (NT::BnaC.DGAT1.a). White circle: NT::BnaA.DGAT1.b. Black down-pointing triangle: BnaC.DGAT1.a. White down-pointing triangle BnaA.DGAT1.b. Black square: LacZ
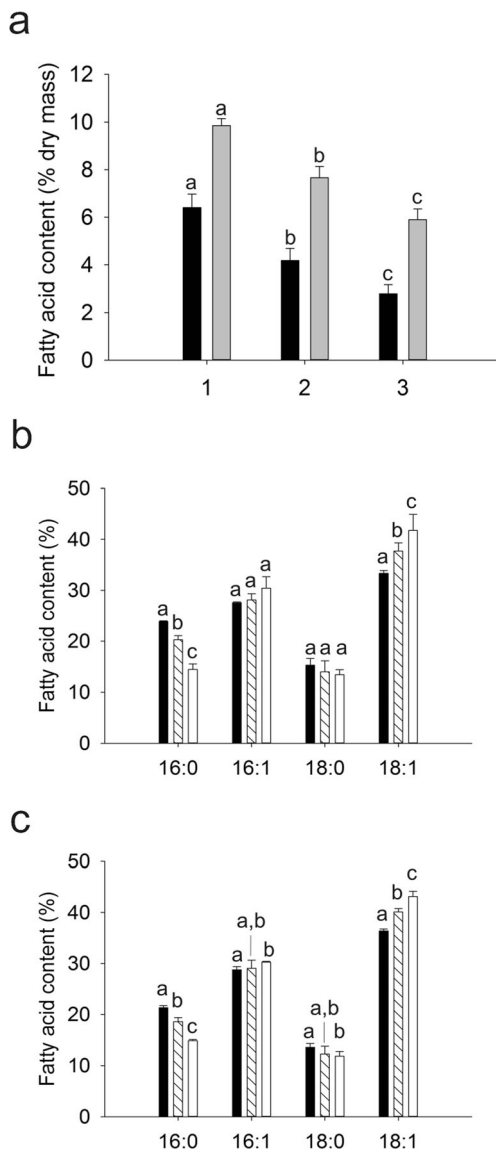
Fig. 6 Fatty acid accumulation (**a**) and composition of triacylglycerol (**b**) and total lipid fractions (**c**) isolated from wild-type *S. cerevisiae* cultures expressing *BnaDGAT1* constructs with and without coding segments for the N-terminal tag 72 h post-induction. In **a**, N-terminally tagged *BnaA.DGAT1.b* (*1*), *BnaA.DGAT1.b*, (*2*) and *LacZ* (*3*) are indicated with *numbers*, and TAG (*black square*) and total lipids (*grey square*) are indicated by *color*. In **b** and **c**, above enzymes are indicated with *black*, *striped*, and *white bars*, respectively. *Error bars* indicate ±1 standard deviation. *Letters* indicate statistical grouping

BnaA.DGAT1.b content, through addition of an N-terminal tag, resulted in increased palmitic acid (16:0) and reduced oleic acid (18:1Δ^{9cis}) content in either lipid fraction. Small differences in palmitoleic (16:1Δ^{9cis}) and stearic acid (18:0) contents were observed, however, between total lipid fractions of tagged *BnaA.DGAT1.b* and *LacZ*-expressing cultures. For the enzyme BnaC.DGAT1.a, which naturally displays relatively high activity, no significant differences in fatty acid

profiles were observed between cultures expressing tagged and untagged versions of this enzyme.

Increasing the raffinose content of the standard media (2 % galactose, 1 % raffinose), while maintaining galactose and nitrogen contents constant, led to a small increase in cellular growth without penalty to neutral lipid accumulation. Under these conditions, the tagged and untagged versions of BnaC.DGAT1.a produced 453 and 370 mg/L of fatty acids, respectively, in 72 h (data not shown). This translates to an increase of 22 % in total lipid content. In the same time period, tagged and untagged versions of BnaA.DGAT1.b produced 416 and 252 mg/L fatty acids, respectively, an increase of 65 %. The wild-type control produced 242 mg/L total fatty acids under the same conditions. Higher concentrations of raffinose were observed to either provide no improvement or to be deleterious.

Discussion

Here, we report the identification of four transcriptionally active *DGAT1* genes from the major oilseed crop *B. napus*. To initially characterize the products of these genes, we expressed their CDSs in *S. cerevisiae*, a model organism commonly used for plant lipid research (Liu et al. 2011; Sandager et al. 2002; Siloto et al. 2009a; Turchetto-Zolet et al. 2011; Yu et al. 2008; Zhang et al. 2013). This initial characterization, however, was hindered by the large differences observed in BnaDGAT1 isoforms' accumulation when produced in this species. We then identified that placement of a tag on the N-termini of the BnaDGAT1 could dramatically increase their in vivo accumulation, ultimately leading to increased TAG accumulation. Interestingly enough, a similar effect was observed by O'Quin et al. (2009), who demonstrated placement of Myc or hemagglutinin epitopes at the N-termini of plant desaturases could increase the accumulation of these polypeptides in *S. cerevisiae*. We probed the mechanism further and observed that the N-terminal tag of pYES2-NT appeared to increase DGAT1 accumulation by masking deleterious polymorphisms present at the +5 nucleotide position of the four *DGAT1* CDSs. It would be interesting to investigate how this polymorphism can account for a 149-fold difference in in vitro activity. Speculatively, it has been demonstrated that the identity of the amino acid residues present at the N-terminus of proteins can significantly influence their turnover rate (Sriram et al. 2011; Tasaki et al. 2012), which is a possibility given that the BnaDGAT1 CDS, and the N-terminal tag, encode different amino acid residues at the N-termini. An alternative possibility may be that this polymorphism affects the rate at which this CDS is translated. Analysis of Kozak sequences from highly expressed yeast genes suggests that the +5 cytosine of BnaA.DGAT1.b and BnaC.DGAT1.b would actually be favored and not

detrimental (Hamilton et al. 1987). A third possibility worthy of future exploration is that N-termini of DGAT1 are involved in a regulatory mechanism specific to this enzyme. Indeed, it has been suggested that DGAT1 may be regulated through the interaction of their N-terminal domains with acyl-CoAs (Weselake et al. 2006; Siloto et al. 2008).

Irrespective to the underlying mechanism, addition of an N-terminal epitope was shown dramatically increase the activity of all four BnaDGAT1 enzyme forms expressed in yeast. Identifying a simple cloning method which can increase the accumulation of at least some DGATs when produced in yeast should be of great value to researchers studying the biochemical properties of these enzymes. It could also have benefits for researchers attempting to increase lipid content in yeast for biofuel and other industrial applications. To our knowledge, there is only one report of in vitro DGAT1 activity using a *S. cerevisiae* extract as high as what we observed with the tagged BnaDGAT1 enzymes (Yu et al. 2008), whereas other reports are of several orders of magnitude lower. The specific activity of microsomal NT::BnaC.DGAT1.a observed here was approximately 11-fold higher than that reported for lipid body fractions containing a genetically improved version of DGA1, despite the latter being expressed in a wild-type background (Kamisaka et al. 2010). In the SCY 62 background, placing an N-terminal tag on BnaA.DGAT1.b resulted in a 67 % increase in total fatty acid content for the DGAT-producing cultures, which corresponded to approximately 10 % of the dry weight of the cells. This level of lipid accumulation is similar to that achieved by overexpressing *DGA1* under the control of the Gal1 promoter in *S. cerevisiae* (Runguphan and Keasling 2014). That study, however, reported a much lower production value of 168 mg/L of fatty acids compared to the 453 mg/L reported here. Kamisaka et al. (2013) reported a similar total fatty acid production value (450 mg/L) when expressing *DGA1* under the control of *ADH1* for 7 days in the presence of 10 % glucose. Thus, it appears that production of tagged BnaDGAT1 enzymes under the control of *GAL1* can provide equivalent or better fatty acid production results relative to those achieved by the overexpression of *DGA1* in *S. cerevisiae*. It should be noted, however, that Kamisaka et al. (2013) were able to achieve a much greater yield of fatty acids (up to 930 mg/L) by expressing an N-terminally truncated *DGA1* in a Δ*dga* background. In light of these findings, it would be of great interest to observe how N-terminally tagged BnaDGAT1 performed when expressed in a Δ*dga* background or when co-expressed with the improved *DGA1* variant.

Although *S. cerevisiae* is known to produce lower lipid content than many oleaginous yeast strains, it has many genetic tools available and is familiar to industry and thus has been suggested to be a good model strain for yeast-based biofuel production (Runguphan and Keasling 2014). Further genetic modification of yeast metabolism to increase substrate production for the tagged DGAT1 enzymes may further maximize the potential effectiveness of these modified TAG-biosynthetic enzymes. The in vitro activity increases observed for these enzymes were significantly greater than those observed for TAG accumulation, and the extracts required addition of exogenous DAG for maximal performance. This suggests that DAG availability may be limiting in the synthesis of TAG.

We noted the fatty acid profiles of both TAG and total lipid fractions were significantly different between cultures expressing tagged and untagged versions of *BnaA.DGAT1.b*. This observation may have important implications for the engineering of yeast to produce desaturated fatty acids, as there was a negative relationship between DGAT1 accumulation and unsaturated fatty acid content. Although the metabolic "pull" created by the catalytic action of DGAT is generally accepted to promote TAG accumulation in yeast, it may be that in certain circumstances that reducing DGAT activity to a moderate level may increase the relative content of unsaturated fatty acids in TAG. Conversely, it may be necessary to upregulate Δ9-desaturase activity or increase the abundance of this enzyme when increasing DGAT accumulation in yeast in order to achieve a higher proportion of unsaturated fatty acids in TAG. Indeed, both DGAT and Δ9-desaturases of yeast are present in the ER (Stukey et al. 1990) and presumably compete for cytosolic acyl-CoA as substrates.

In summary, placement of an N-terminal epitope on DGAT1 enzymes can provide a facile method to eliminate the influence of native N-terminal sequences during heterologous expression in *S. cerevisiae*. This discovery could potentially benefit researchers involved in studying the biochemical properties of recombinant DGAT produced through heterologous expression in yeast and has implications for optimization of plant *DGAT* to facilitate effective production of the encoded recombinant polypeptides in this microbial organism. In addition, this discovery offers a new approach to increase lipid content in yeast for industrial applications such as producing feedstock for biodiesel production.

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